Immunogenetics (2001) 52: 231–241
DOI 10.1007/s002510000267

ORIGINAL PAPER

Stefan Rothenburg · Friedrich Koch-Nolte
Heinz-Günter Thiele · Friedrich Haag

DNA methylation contributes to tissue- and allele-specific expression of the T-cell differentiation marker RT6

Received: 21 August 2000 / Revised: 17 October 2000 / Published online: 13 December 2000
© Springer-Verlag 2000

Abstract We investigated the role of DNA methylation in gene regulation of the rat T-cell differentiation marker RT6. Analysis of the methylation status of various tissues revealed that the *RT6* promoter was hypomethylated in *RT6*-expressing tissues, and methylated in nonexpressing ones. Remarkably, among *RT6*-nonexpressing tissues, the extent of methylated regions varied greatly between lymphatic tissues, where regions larger than 23 kb were methylated, and nonlymphatic tissues, where methylation was restricted to a 3- to 4-kb region surrounding the promoter. We have previously shown that *cis*-regulatory elements determine differential expression of the two *RT6* alleles in a subpopulation of T cells. We now show that the *RT6* alleles in these cells differed in their methylation status. The promoter region of the silent allele was methylated, while that of the transcribed allele was not. Upon treatment of *RT6*-nonexpressing thymoma cells with the methyltransferase inhibitor 5-azacytidine, *RT6* expression was induced. In *RT6* heterozygous hybridoma cells, expressing only one *RT6* allele, induction of the silent, methylated *RT6* allele was observed. Sensitivity of the *RT6* promoter to DNA methylation was demonstrated by promoter-specific in vitro methylation, which inhibited *RT6* promoter activity, while that of the SV40 promoter was not influenced. Our findings indicate that DNA methylation plays an important role in the control of monoallelic and tissue-specific *RT6* expression.

Keywords RT6 · T-cell development · DNA methylation · Tissue specificity · Monoallelic expression

S. Rothenburg · F. Koch-Nolte · H.-G. Thiele · F. Haag (✉)
Institute for Immunology, University Hospital Eppendorf, 20246 Hamburg, Germany
E-mail: haag@uke.uni-hamburg.de
Phone: +49-40-428034595
Fax: +49-40-428034243

Introduction

Expression of the rat T-cell differentiation marker RT6 is restricted to lymphocytes. Within this lineage, only mature peripheral T cells, intraepithelial lymphocytes, and a subpopulation of natural killer cells express this protein (Fangmann et al. 1991; Thiele 1988; Wonigeit et al. 1997). RT6 is not expressed in thymocytes or recent thymic migrants (Thiele 1988).

RT6 is an NAD-metabolizing, glycosyl phosphatidylinositol-linked ectoenzyme which belongs to the family of mono-ADP-ribosyltransferases (ARTs). Other ARTs have recently been described in lymphocytes, spleen, muscle, and testes of both humans and rodents (reviewed in Haag and Koch-Nolte 1998; Okazaki and Moss 1998). In the mouse, mono ADP-ribosylation has been shown to suppress T-cell function and the activity of the protein tyrosine kinase p56lck. Target proteins identified so far include LFA-1 (CD11a/CD18), CD8, CD27, CD43, CD44, and CD45 (Nemoto et al. 1996; Okamoto et al. 1998). In the BB rat model for insulin-dependent diabetes mellitus, defective RT6 expression coincides with susceptibility to the disease (Greiner et al. 1987). Inactivating mutations of the mouse homologue Rt6 (Art2) have been found in NZW and B×SB mice, strains associated with two independent models for systemic lupus erythematosus (Koch-Nolte et al. 1995; Matthes et al. 1997).

In the rat, *RT6* is a single-copy gene that contains eight exons. Two independent promoters have been identified, of which promoter 2 is the main promoter in adult rat spleen cells (Haag et al. 1996). RT6 is encoded by the two highly divergent alleles *RT6a* (coding for RT6.1) and *RT6b* (coding for RT6.2). These alleles display only 95% homology at the protein level and differ in ten amino acids with mostly nonconservative exchanges (Haag et al. 1990a; Koch et al. 1990). The expression of these alleles is distinctively regulated. (*RT6a*×*RT6b*) F1 rats express both

alleles on the majority of mature T cells but contain a subpopulation, accounting for approximately 30% of cells, in which only RT6.2 is expressed. We have previously shown that this monoallelic expression is due to a *cis*-acting pretranslational mechanism (Thiele et al. 1993). We looked for differences between the two *RT6* alleles that may explain the monoallelic expression of this antigen in T cells, and identified sequence variations in the promoter regions of the *RT6* alleles. Reporter studies revealed that the promoter of *RT6a* was twice as strong as that of *RT6b* in an *RT6*-expressing cell line, making it unlikely that these differences contribute to monoallelic expression of RT6.2 (Rothenburg et al. 1999a). We have also demonstrated a correlation between DNA methylation and *RT6* gene activity in rat cell lines, raising the question whether this modification is important for the control of the tissue and allele specificity of *RT6* expression (Rothenburg et al. 1999b).

DNA methylation is a key player in the regulation of many genes (reviewed by Mostoslavsky and Bergman 1997; Tajima and Suetake 1998). A main function for DNA methylation in vertebrates may be to reduce transcriptional noise (Bird 1995). Methylation is also thought to function as a host defense mechanism against foreign and intragenomic parasites (Doerfler 1991; Walsh and Bestor 1999; Yoder et al. 1997). The inhibitory effects of methylation on transcription are mediated by changes in the binding affinity of proteins to their target sequences (Ehrlich and Ehrlich 1993); many transcription factors have been found to be sensitive to DNA methylation, binding only to their target sequence if it is not methylated (reviewed in Ehrlich and Ehrlich 1993; Klempnauer 1993; Prendergast et al. 1991). Additionally, several methylcytosine-binding proteins have been identified, some of which have the potential to repress transcriptional activity, probably through the attraction of histone deacetylases (reviewed in Newell-Price et al. 2000).

The study presented here investigated whether DNA methylation is important in the regulation of tissue-specific *RT6* expression and whether differences in the methylation status of each allele contribute to monoallelic *RT6* expression.

Materials and methods

Animals and cell lines

LEW.6B (*RT6b*) rats were originally kindly provided by K. Wonigeit (Hannover), and are maintained at the Animal Care Facility of the University Hospital. BDE (*RT6b*) rats were purchased from the Institute for Laboratory Animal Science, Medical School Hannover, Germany. LEW and dpBB (both *RT6a*) rats were originally purchased from Møllegaard (Skensved, Denmark) and are maintained in the Animal Care Facility of the University Hospital. *RT6* heterozygous animals were obtained by mating LEW and LEW.6B or dpBB and BDE rats. C58NT thymoma cells were kindly provided by M. Nabholz. Rat1 fibroblasts were kindly provided by M. Wegner (Hamburg). Construction of EpSM30 cells (Haag et al. 1990a) and EpD3 cells (Koch et al. 1988) has been described. C58NT, EpSM30, and EpD3 cells were grown in RPMI medium containing 10% fetal calf serum (FCS; Life Technologies, Karlsruhe, Germany). Rat1 fibroblasts were grown in DMEM medium containing 10% FCS. Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

Cell separation

B cells were depleted from lymph nodes of *RT6* heterozygous (LEW×LEW.6B) and (dpBB×BDE) rats by panning with a polyclonal goat anti-rat Ig antibody. Cells were then selected for RT6.1 expression using the allotype-specific antibody 3G2 and magnet-activated cell sorting (MACS; Milteny). Cells positive for RT6.1 also express RT6.2. The remaining RT6.1-negative cells were subsequently incubated with the RT6.2-specific antibody Gy1/12 and selected for RT6.2 expression.

Cloning the RT6 gene

Genomic DNA from thymocytes and liver cells of LEW.6B rats was PCR amplified using primers derived from the *RT6* gene isolated from a Sprague-Dawley rat genomic DNA library (Haag et al. 1996). DNA polymerases used were AmpliTaq Gold (PE Applied Biosystems, Weiterstadt, Germany), Expand Long (Boehringer Mannheim, Mannheim, Germany) and Taq Plus (Stratagene, Heidelberg, Germany). Amplification products were cloned into the pCR2.1 vector (Invitrogen, Groningen, Netherlands) using the TA-cloning kit or, for long PCR products, TOPO Cloning Kit (both Invitrogen), and sequenced using the dye terminator protocol (PE Applied Biosystems) according to the manufacturer's instructions. More than 80% of sequences were obtained from two or more independent PCR amplifications. The sequences reported in this paper have been deposited in the EMBL database and assigned the accession number AJ297708.

DNA preparation and Southern blot hybridization

Genomic DNA was prepared using the Blood and Cell Culture DNA Mini and Midi Kits (Qiagen, Hilgen, Germany) according to the manufacturer's protocol. DNA (10 μg) was digested with 200 units *Msp*I or *Hpa*II (both Boehringer Mannheim) in the presence of 5 ng RNase and 1 mM spermidine for 2 h at 37 °C. Products were separated on 0.8% agarose gels overnight and blotted to nylon membranes. Membranes were hybridized with a 401-bp fragment amplified from the *RT6* promoter using primers AFC (5′-GGC CGA GGG AGA TAA TGG GTT C-3′) and MER (5′-CGA TAC TGG AGA AGC AGA AGT TGG-3′) or with a 337-bp fragment from the nucleolin gene amplified using primers Ncl 3f (5′-CCC GCC CTC AAA AGC AGA C-3′) and Ncl 1r (5′-TTA CGG CGA CGG CGT GTG TAG A-3′) and radiolabeled with ^{32}P-dCTP using the Rediprime DNA labeling kit (Amersham Pharmacia, Freiburg, Germany).

Methylation-sensitive PCR

DNA of RT6.1+/RT6.2+, RT6.1–/RT6.2+ or EpSM30 cells was digested with restriction endonucleases *Msp*I, *Hpa*II or *Rsa*I (40 units/μg DNA, all Boehringer Mannheim) for 2 h at 37 °C. PCR reactions were performed using 0.2 μg DNA from digests, Ampli-Taq gold (PE Applied Biosystems) and primers PF3 (5′-CCA ACT TCT GCT TCT CCA GTA TCG-3′) and A3R (5′-CTG AGA AGG TGT TTG GCA GC-3′) spanning the polymorphic dinucleotide repeat and the *Msp*I/*Hpa*II site (see Fig. 1). PCR reactions were performed with initial heat activation for 8 min at 94 °C, followed by 27 cycles of 1 min 94 °C, 1 min 60 °C, 40 s 72 °C, and a final extension step of 72 °C for

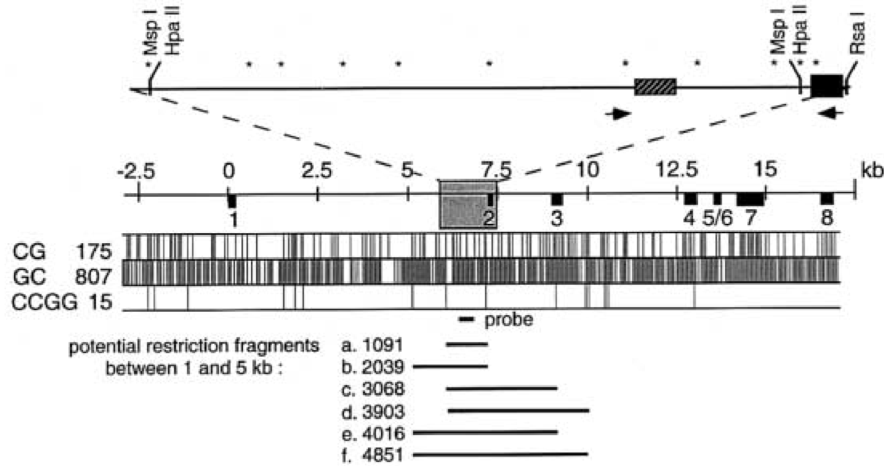


Fig. 1 Map of the *RT6* gene. The *RT6* gene consists of eight exons (*black boxes*) spanning 17 kb. Distribution of CG, GC, and CCGG (*Msp*I/*Hpa*II) sites are shown. The main *RT6* promoter region (*gray box*) is enlarged. The *striped box* shows the location of the polymorphic dinucleotide repeat. Restriction sites used in the assays described in this article, the position of CpG sites (*asterisks*) and the location of primers (*arrows*) used in the methylation-sensitive PCR assay are depicted. The probe used for Southern blot analysis, as well as restriction fragments between 1 and 5 kb, which are detected by it, are indicated. *a* denotes the product detected in digests with *Msp*I, and also in *Hpa*II digests if the CCGG site in the promoter is not methylated. *b–f* denote fragments detected in *Hpa*II digests if the *RT6* promoter region is methylated to different extents

6 min. Location of primers and restriction sites are depicted in Fig. 1. PCR products were visualized on ethidium bromide-stained 2% agarose gels.

5-Azacytidine treatment and expression analysis

EpSM30 and C58NT cells were incubated in RPMI/10% FCS containing 10 μM 5-azacytidine (Sigma, Deisenhofen, Germany). To provide stable conditions, 5-azacytidine was replenished every 24 h. Treated and untreated control cells were harvested at day 6, total RNA was prepared using the RNeasy kit (Qiagen), and surface expression was measured by flow cytometry with biotinylated monoclonal antibodies 3G2 (RT6.1) and Gy1/12 (RT6.2) and streptavidin-fluorescein isothiocyanate.

Reverse transcription-polymerase chain reaction

RNA (1 μg) from the indicated cell lines was primed with random hexamers (Amersham Pharmacia) and reverse transcribed using SuperScript MMLV reverse transcriptase (Life Technologies) according to the manufacturer's instructions. PCR amplification was performed with initial heat activation for 8 min at 94 °C followed by 36 cycles of 1 min 94 °C, 1 min 55 °C, 50 s 72 °C, and a final extension step of 72 °C for 6 min, using *RT6*-specific primers vRAG (5′-GGT TTC AAT GAT TTC CAT GGA ACG GCT TTA GTT GCC TA-3′) and Rs1 (5′-AGA ACC ACA AGG AAC AGG GAT ACA CA-3′) and hypoxanthine guanine phosphoribosyl transferase (HPRT)-specific primers HPF (5′-CCC AAA ATG GTT AAG GTT GCA AGC TTG-3′) and HPR (5′-ACT ARG YAG ATG GCC ACA GGA CTA G-3′). Aliquots of *RT6*-specific PCR reactions were digested with 10 units *Mbo*I (MBI, St. Leon-Rot, Germany) for 1 h at 37 °C. Amplification products were visualized on a 1.6.% agarose gel.

Plasmid construction

A 293-bp fragment from the main *RT6* promoter was PCR amplified with primers DMS (5′-GAA TCA GGA GTT CAG GTT ATC ATC-3′) and A3R, cloned into the pCR2.1 vector and subcloned into the pGL3-Basic luciferase vector (Promega, Mannheim, Germany).

In vitro methylation

Methylation was carried out with *Hpa*II and *Sss*I methylases (New England Biolabs, Frankfurt, Germany) under reaction conditions recommended by the manufacturer. In mock-methylation reactions, S-adenosylmethionine was omitted. One unit methylase/μg DNA was used, and incubation was carried out overnight. Completeness of methylation was monitored by digestion with *Hpa*II. After methylated DNA was completely protected from *Hpa*II digestion, reactions were carried out for an additional 2 h to ensure complete methylation. Promoter-specific methylation was carried out as described by Hohn and co-workers (1996) with the following modifications: the plasmids pGL3-Basic, pGL3-Promoter (Promega), RT6-241/+52 were digested with *Sac*I and *Hind*III (both MBI). Restriction fragments of SV40 and *RT6* promoters and pGL3-Basic vector were recovered from 1.6% (SV40 and *RT6* promoter) and 1% (pGL3-Basic vector) TAE agarose gels using the Jetsorb gel extraction kit (Genomed, Bad Oeynhausen, Germany), and methylated/mock-methylated with *Sss*I as described above. Ligations of promoter fragments into the pGL3-Basic vector were carried out overnight using T4 DNA ligase (Invitrogen) and analyzed on an agarose gel after *Hpa*II digestion. Methylation and ligation reactions were ethanol precipitated before transfection.

Transient transfection

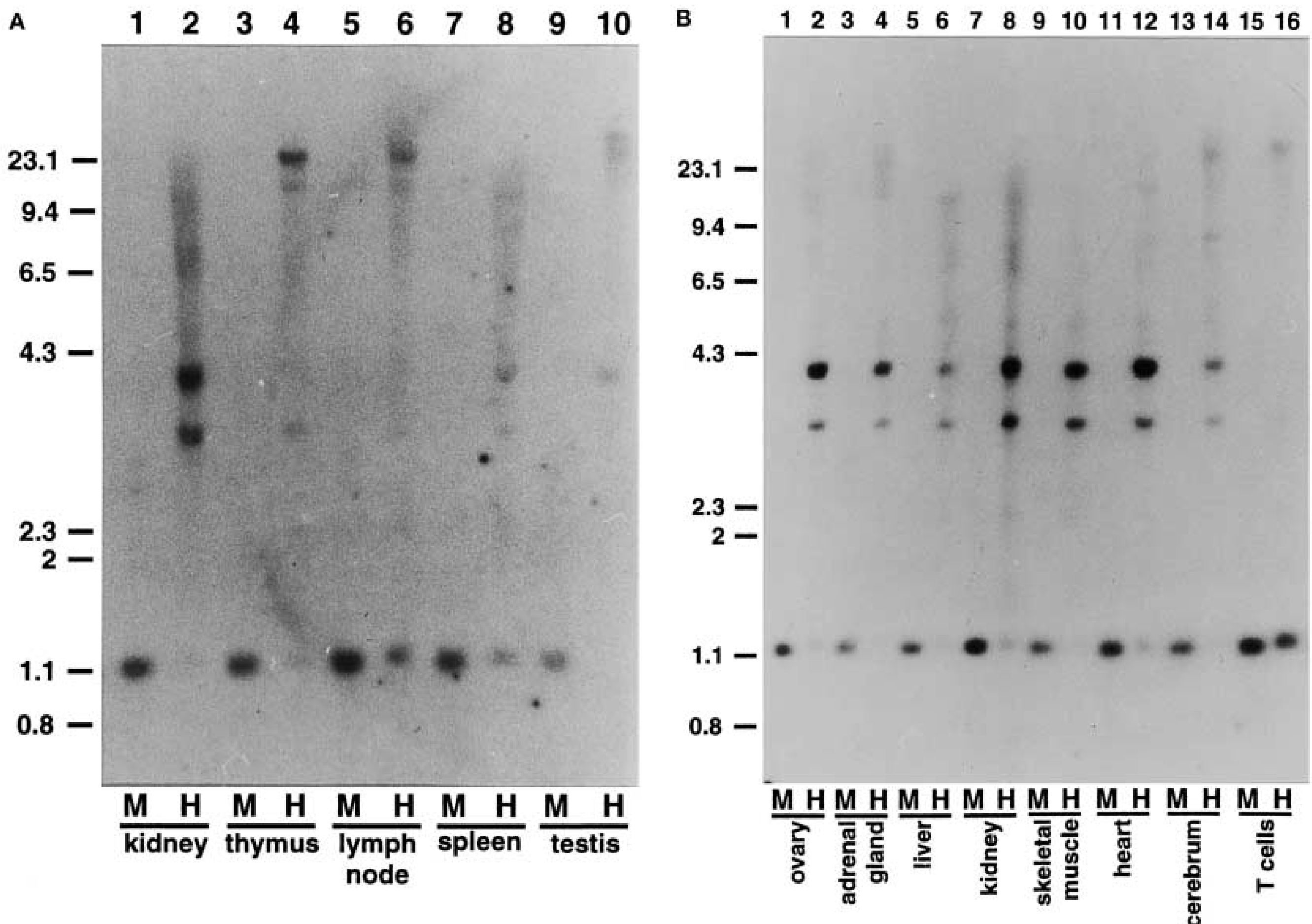
EpSM30 (7×10^6 cells), EpD3 (7×10^6), and Rat1 fibroblasts (2×10^6 cells) were transfected by electroporation (0.3 V/960 μF) with 10 μg plasmid DNA in 700 μl RPMI medium containing 10% FCS using a Gene pulser electroporator (Bio-Rad, Munich, Germany). Cells were immediately resuspended in 10 ml fresh RPMI/10% FCS and analyzed after 46–48 h for luciferase activity using the luciferase assay (Promega) according to the manufacturer's instructions in a luminometer, kindly shared by M. Wegner (Hamburg).

Results

Demethylation of the RT6 promoter region is correlated with RT6 expression

We sequenced the complete *RT6* gene, including the 5′ and 3′ regions, from genomic DNA of LEW.6B rats, which carry the *RT6b* allele of the BH rat on the LEW background (Wonigeit 1979). The *RT6* gene spans circa 17 kb (Fig. 1). The overall CpG/GpC ratio is 0.22 and no evidence for CpG islands was found. We analyzed the methylation status of the *RT6* promoter by digesting genomic DNAs from various tissues with the methylation-resistant endonuclease *Msp*I or the methylation-sensitive endonuclease *Hpa* II followed by Southern blot hybridization (Fig. 2A, B). A 401-bp PCR-amplified fragment was used as a probe for the promoter region. Potential restriction fragments in the range between 1 and 5 kb detected by this probe are depicted in Fig. 1. Fragment a is generated by *Msp*I digestion, and is identical to the product of *Hpa*II digests if the promoter is not methylated. Fragments c–f denote products expected if the *Hpa*II site 16 bp 5′ to the transcription start site is methylated, but surrounding sites are not. In tissues where *RT6* was not expressed (confirmed by RT-PCR, not shown), no 1.1-kb bands were observed in *Hpa*II digests, indicating promoter hypermethylation. In *Hpa*II digests of RT6-expressing total lymph node (Fig. 2A, lane 6), spleen (Fig. 2A, lane 8), and lymph node T cells (Fig. 2B, lane 16), 1.1-kb bands were readily visualized, demonstrating that the promoter region was hypomethylated in a considerable number of these cells. Intriguingly, varying degrees of methylation can be observed when different *RT6*-nonexpressing tissues are compared. In thymocytes and testis as well as in the methylated fraction of lymph node and purified T cells, high-molecular-weight bands above 23 kb in the *Hpa*II digests indicate that extended regions of the *RT6* gene were methylated. In all other tissues, prominent bands were seen at 3 and 4 kb, corresponding to fragments c and d/e, respectively. The

Fig. 2A,B Methylation status of the *RT6* gene in various tissues determined by Southern blot analysis. Genomic DNA (10 μg) from the indicated tissues of 70- to 90-day-old LEW.6B rats was digested with either methylation-resistant *Msp*I (*M*) or its methylation-sensitive isoschizomer *Hpa*II (*H*), separated on 0.8% agarose gels, and blotted to nylon membranes. The membranes were hybridized with a 401-bp PCR-amplified fragment from the promoter region, which is depicted in Fig. 1. The two kidney samples in **A** and **B** are derived from different animals. The positions of DNA molecular-weight markers (in kb) are indicated



latter bands could only be generated if the promoter *Hpa*II site was methylated and surrounding sites were not, indicating that methylation in these nonlymphoid organs was restricted to the promoter region. The faint 1.1-kb bands observed in *Hpa*II digests of kidney and heart are probably due to contaminating *RT6*-expressing blood lymphocytes. The same blots were also hybridized with a fragment from the promoter of the rat nucleolin gene. This gene is ubiquitously transcribed and is part of a CpG island (Bourbon et al. 1988). It is thus not expected to be methylated. A 608-bp fragment, corresponding to the predicted size after successful *Msp*I/*Hpa*II digestion, was detected equally in *Msp*I and *Hpa*II digests of all tissues, demonstrating hypomethylation of this gene and complete DNA digestion (not shown).

The silent allele in monoallelically RT6-expressing cells is methylated

We tested whether the general correlation observed between methylation status and *RT6* expression also applied to the asymmetric expression of *RT6* alleles in a T-cell subpopulation of *RT6* heterozygous rats. Such rats express both RT6 allotypes on the majority of mature peripheral T cells ('double-positive' cells). A subpopulation of mature T cells, accounting for circa 30%, expresses only RT6.2 but not RT6.1 ('single-positive' cells) (Fig. 3A). Both subpopulations were isolated from lymph nodes of F1 offspring of LEW (*RT6a*) and congenic LEW.6B (*RT6b*) rats, which are genetically identical except for their *RT6* locus (Wonigeit 1979) and DNAs were prepared. The purity of single-positive and double-positive cells as determined by FACS analysis was >90% (not shown). Methylation-sensitive *Hpa*II PCR assays (Singer-Sam et al. 1990) and control PCRs were performed using primers and restriction sites as indicated in Fig. 1. Prior to amplification, DNAs were digested with *Msp*I, *Hpa*II, or *Rsa*I, the latter of which does not cleave within the PCR template. The two alleles can be discriminated by a polymorphic dinucleotide repeat within the amplified fragment (Haag et al. 1997).

Comparison of the amplification products obtained from *Hpa*II-treated versus *Rsa*I-treated DNA from double-positive cells showed a marked reduction in the intensity of the bands corresponding to both alleles (Fig. 3B, lanes 5, 6), indicating that in these cells, both alleles were hypomethylated. In contrast, in single-positive cells, a corresponding decrease was seen only for *RT6b*, while no change was observed for the silent *RT6a* allele, which thus was apparently fully methylated (Fig. 3B, lanes 2. 3). In *Msp*I digests (lanes 1, 4), no bands were seen, indicating complete digestion of the templates. The faint bands that are visible in *Hpa*II digests are probably due to contamination by *RT6*-nonexpressing cells that were not removed by the cell separation. Control PCRs using primers specific

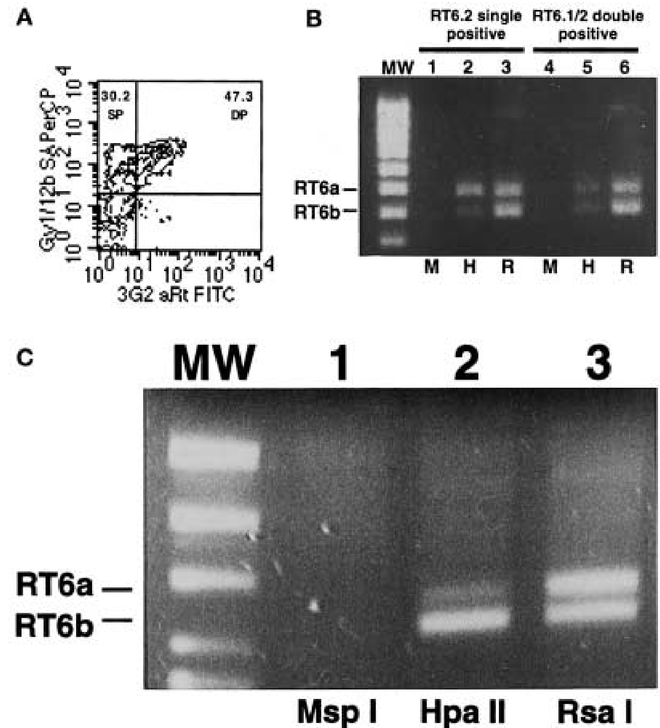


Fig. 3A–C Methylation status of the *RT6* alleles in monoallelically *RT6*-expressing cells. **A** Immunostaining with RT6 allotype-specific antibodies of lymph node T cells from F1 animals. Percentages of single-positive (*SP*) cells, detected only by the RT6.2-specific antibody Gy1/12, and of double-positive (*DP*) cells, also detected by the RT6.1-specific antibody 3G2 are indicated. **B** Genomic DNA was prepared from the sorted cell populations and digested with *Msp*I, *Hpa*II, or *Rsa*I (40 units/µg DNA). PCR amplifications spanning the *Msp*I/*Hpa*II site were performed as indicated in Fig. 1. Products were visualized on a 2% agarose gel. Amplification products representing the two alleles can be discriminated due to a polymorphic dinucleotide repeat. DNA digested with *Rsa*I, which cuts outside the PCR template, was used as a control for amplification (*lanes 3, 6*). DNA digested with *Msp*I was used as a control for complete digestion (*lanes 1, 4*). PCR amplification of DNA digested with *Hpa*II, which is methylation-sensitive, only produces products if the *Hpa*II site is methylated and therefore protected. The expected sizes of PCR products representing the two *RT6* alleles are indicated. The data shown are representative of several independent experiments using DNA from different heterozygous rats. **C** Genomic DNA from the T-cell hybridoma EpSM30, which is *RT6* heterozygous but only expresses RT6.1, was assayed as described above

for a fragment of the *RT6* gene that does not contain any target site for *Msp*I/*Hpa*II or *Rsa*I showed comparable amounts of amplification products from all digests (data not shown). Similar results were obtained for T cells from *RT6* heterozygous F1 and backcross animals of dpBB (*RT6a*) and BDE (*RT6b*) rats (data not shown).

We also investigated the association of methylation and *RT6* expression in another case of monoallelic *RT6* expression, the T-cell hybridoma EpSM30 (Fig. 3C). This cell line was created by fusion of the *RT6*-nonexpressing thymoma C58NT, which carries

the *RT6b* allele, with spleen cells from a LEW rat, which carries the *RT6a* allele. EpSM30 is genetically heterozygous (Haag et al. 1997) but transcribes only the *RT6a* allele. Eighty percent of EpSM30 cells used for DNA preparation expressed RT6.1 while none expressed RT6.2 (not shown). Methylation-sensitive PCR demonstrated that the silent *RT6b* allele was hypermethylated, whereas the transcribed *RT6a* allele was hypomethylated, indicated by the strong and faintly visible bands, respectively (Fig. 3C, lane 2). The residual band observed for *RT6a* is probably due to the presence of the *RT6*-nonexpressing cells.

5-Azacytidine induces expression of the silent RT6b allele in EpSM30 cells

To further investigate the importance of methylation for *RT6* expression in heterozygous cells, we tested whether inhibition of methylation can induce *RT6* expression in the *RT6*-nonexpressing thymoma cell line C58NT, and expression of the silent allele in EpSM30 cells, which express *RT6* monoallelically. EpSM30 and C58NT cells were incubated with the methyltransferase inhibitor 5-azacytidine or left untreated (control). Six days after incubation, we analyzed expression of *RT6* alleles by flow cytometry. Figure 4B shows induction of RT6.2 expression in C58NT (genotypically *RT6b*) cells from background levels on day 0, to 11% of cells on day 6. As expected, in C58NT cells, no RT6.1 expression was observed at any time (Fig. 4A). The silent RT6.2 allele of EpSM30 cells was induced from background levels at day 0 to 30% at day 6 (Fig. 4D). Concomitantly, the number of RT6.1-expressing cells increased slightly from 56 to 73% (Fig. 4C). Cell surface expression of proteins analyzed was verified by fluorescence microscopy. The number of EpSM30 cells expressing RT6 increased continuously over the 6-day period of 5-azacytidine treatment (Fig. 4E).

To confirm that methyltransferase inhibition induced transcription of *RT6*, we assayed for *RT6* mRNA in 5-azacytidine-treated cells. We performed RT-PCR using primers spanning exons 7 and 8. The template includes an *Mbo*I site, which is present in *RT6b* but absent in *RT6a*. This enzyme can be used to distinguish between the amplification products from the two alleles. No *RT6b*-specific bands appeared in the *Mbo*I digest of untreated EpSM30 cells (Fig. 5, lane 3), indicating that in these cells, only the *RT6a* allele was transcribed. Induction of the silent *RT6b* allele by 5-azacytidine treatment is demonstrated by the appearance of two *RT6b*-specific bands at 291 and 239 bp (Fig. 5, lane 6). Control RT-PCR of RNA from EpD3 cells, which carry two distinct *RT6b* alleles, showed complete digestion of the PCR product as expected (lane 9). No *RT6*-specific band could be observed in untreated C58NT cells (lanes 10, 12). Upon 5-azacytidine treatment, a band was detected (lane 13), which was completely digested by *Mbo*I (lane 15), thus demonstrating induction of *RT6b* transcription in these cells. Additional PCR products were generated in 5-azacytidine-treated C58NT cells that were not digested by *Mbo*I (lanes 13, 15), which most likely represent unspecifically amplified genes induced upon methyltransferase inhibition.

Inhibition of RT6 promoter activity by in vitro methylation

To test whether in vitro methylation influences *RT6* promoter activity, we methylated luciferase reporter gene plasmids (pGL3) carrying either a fragment of the *RT6* promoter, which shows no difference between

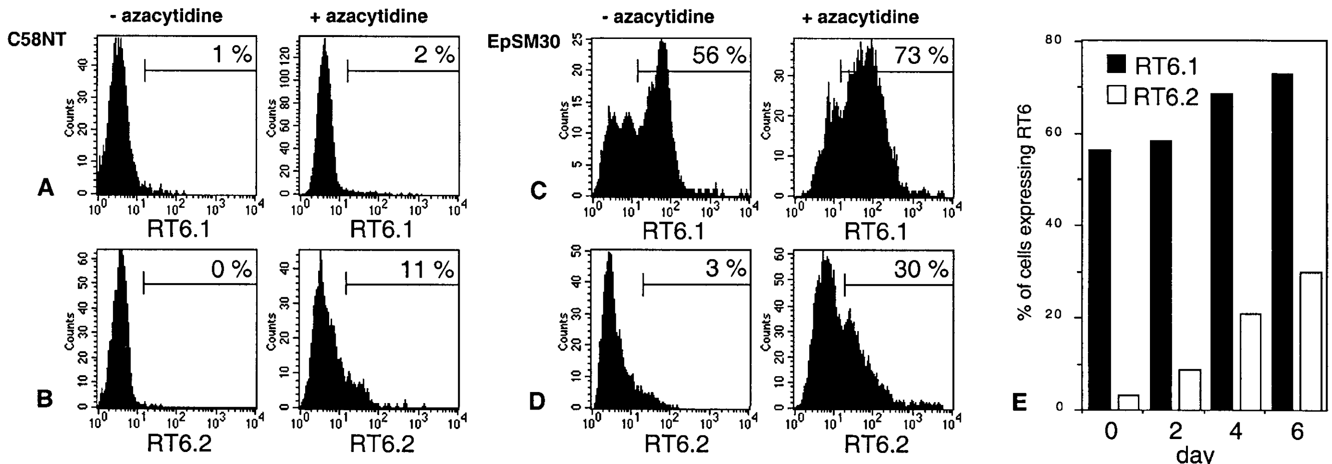


Fig. 4A–E Flow cytometric analysis of RT6 expression of 5-azacytidine-treated cells. **A–D** C58NT and EpSM30 cells were grown in the absence or presence of 5-azacytidine for 6 days and were stained with monoclonal antibodies 3G2 (RT6.1) and Gy1/12 (RT6.2). The percentage of positive cells is indicated in each case. **E** Percentage of EpSM30 cells expressing RT6.1 (*black bars*) or RT6.2 (*white bars*) over a 6-day period upon incubation with 5-azacytidine. Results shown are representative of three independent experiments

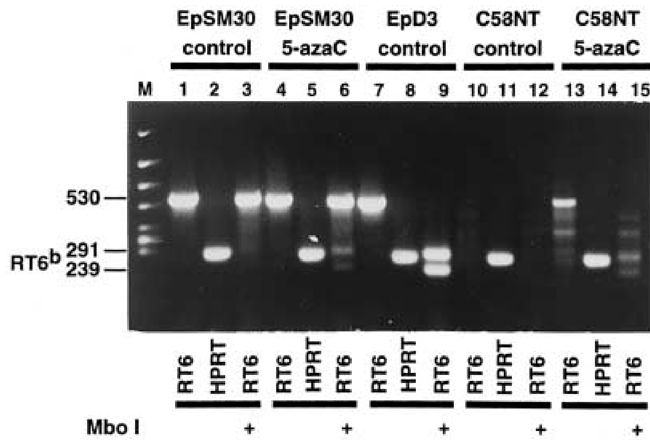


Fig. 5 RT-PCR from 5-azacytidine (*azaC*)-treated cells. RNA was prepared from 5-azacytidine-treated (EpSM30 and C58NT) and untreated (EpSM30, C58NT, and EpD3) cells. RT-PCR products were separated on a 1.6% agarose gel. RT6-specific PCR was performed with primers spanning exons 7 and 8 (*lanes 1, 4, 7, 10, 13*). Aliquots of RT6-specific PCR reactions were digested with *Mbo*I, which recognizes a single base polymorphism in exon 7 and cuts only *RT6b* transcripts (*lanes 3,6,9,12, 15*). The mRNA level of the housekeeping gene *HPRT* was determined as a control (*lanes 2, 5, 8, 11, 14*). The sizes of uncut *RT6* transcripts (530 bp) or *RT6b*-specific *Mbo*I digestion products (291 and 239 bp) are indicated

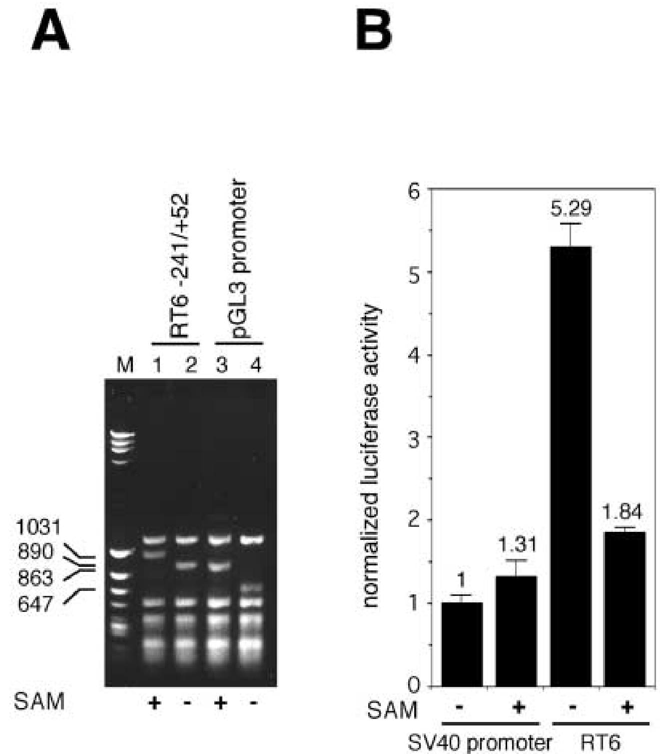


Fig. 6A,B Effect of methylation on *RT6* promoter activity. **A** Excess *Hpa*II endonuclease (40 units/μg DNA) was used to determine the methylation status of plasmids used for transfections. Products were visualized on a 1.2% agarose gel. Successful ligation and methylation of promoter-specific reactions is shown in *lanes 1–4*. Size markings indicate expected sizes of fragments derived from successful ligations, either *Hpa*II-resistant (1031 bp/*RT6*, 863 bp/pGL3 promoter), or *Hpa*II-sensitive (890 bp/*RT6*, 647 bp/pGL3 promoter). Methylation or mock-methylation was carried out in the presence or absence of SAM (indicated by + or –). **B** EpSM30 cells were transfected with 10 μg plasmid. Luciferase activity was assessed after 48 h. The activity of the mock-methylated SV40 promoter was set at 1. *Error bars* show SDs from three independent transfections

the *RT6* alleles, or the SV40 early promoter with either *Hpa*II or *Sss*I methyltransferase. Both methyltransferases dramatically reduced promoter activities of both plasmids (not shown). Since the SV40 promoter does not contain any target sites for *Hpa*II methyltransferase, we surmise that promoter-specific methylation may not be the sole reason for the observed reduction of promoter activities. Indeed, methylation in the coding regions of reporter plasmids or noncoding regions has been shown to be sufficient to inhibit transcription in reporter assays, probably due to interference with transcript elongation or chromatin formation (Graessmann and Graessmann 1993; Hohn et al. 1996; Singal et al. 1997).

We therefore selectively methylated the promoter regions of the above-mentioned plasmids and ligated them into the promoterless pGL3-Basic vector. Successful methylation and ligation was demonstrated by digestion with *Hpa*II (Fig. 6A). Digestion of the methylated *RT6* construct (lane 1) generated a 1031-bp band, which was shifted to 890 bp in the mock-methylated digest (lane 2). Methylation of the SV40 promoter region is indicated by protection of an *Hpa*II restriction site 5′ of the SV40 promoter yielding an 863-bp band (lane 3), which was shifted to 647 bp if mock-methylated (lane 4). Successful ligation is demonstrated by the appearance of the described bands. The 1.2-kb band visible in all four *Hpa*II digests results from unproductive vector-to-vector ligation. In Fig. 6B, the results of transfections of EpSM30 cells are shown normalized for the luciferase activity of the mock-methylated SV40 promoter. The methylated SV40 promoter (bar 2) showed no significant difference compared to the mock-methylated promoter (bar 1). A 2.8-fold decrease in promoter activity was observed when the *RT6* promoter region was methylated (bars 3, 4). Results shown are representative of two experiments, where methylation and ligation reactions as well as transfections were performed independently. It is noteworthy that the fragment of the *RT6* promoter used in these experiments has only four target sites for the *Sss*I methyltransferase, while the SV40 promoter, which was not affected by methylation, has ten.

Discussion

The results presented here indicate that DNA methylation plays a pivotal role in the control of *RT6* expression. The observed CpG/GpC ratio of 0.22 and lack of CpG islands is typical of tissue-specific genes that are

thought to be regulated by DNA methylation (Antequera and Bird 1993). We observed a general correlation of *RT6* gene activity with methylation status in the *RT6* promoter, i.e., the promoter is not methylated in *RT6*-expressing tissues and is methylated in nonexpressing tissues. A similar correlation has been reported for a number of tissue-specific genes (Yeivin and Razin 1993). Remarkably, differences in the extent of the methylated regions are found when *RT6*-nonexpressing cell populations are compared. In most nonlymphatic tissues investigated, methylation was restricted to a region immediately surrounding the promoter. The sole exception was testis, which represents a special case due to the presence of high amounts of gametes (Jaenisch 1997; Walsh and Bestor 1999). In contrast, in *RT6*-nonexpressing cells of lymphatic origin, i.e., thymocytes and a subset of lymph node cells and T cells, regions greater than 23 kb surrounding the promoter were methylated. These findings support the hypothesis that a main function of DNA methylation is repression of inappropriate gene transcription (Bird 1995). We propose that in lymphatic tissues, where the sets of transcription factors are similar (Clevers and Grosschedl 1996), successful repression of *RT6* transcription requires methylation of large regions of the gene, including enhancer regions. In contrast, in nonlymphoid tissues, which lack lymphocyte-specific transcription factors, methylation of the immediate promoter region is sufficient for effective transcriptional repression.

During development from thymocytes to mature T cells, the *RT6* gene must be demethylated for transcription to occur. This is modeled in our experiments, which demonstrated the induction of RT6.2 expression in C58NT and EpSM30 cells upon 5-azacytidine treatment, and thus confirm the role of DNA methylation in *RT6* expression. The results of methylation-sensitive PCR of monoallelically *RT6*-expressing cell populations reveal a striking relationship between expression and methylation status of the alleles. In the RT6.2 single-positive T-cell population of heterozygous rats, the transcribed *RT6b* allele was not methylated while the silent *RT6a* allele was. In contrast, both alleles were unmethylated in cells expressing both alleles. This is consistent with the results obtained from Southern blot analysis of LEW.6B T cells. In EpSM30 cells, the silent *RT6b* allele was methylated as it is in the parental C58NT cells (Rothenburg et al. 1999b). In this cell line, methylation status has apparently been firmly inherited over hundreds of generations. It has been argued that demethylation of tissue-specific genes might be a secondary effect following transcriptional activation by specific transcription factors (Walsh and Bestor 1999). This obviously does not apply for the *RT6* gene. The silent alleles in RT6.2 single-positive F1 T cells and in EpSM30 cells were methylated even in the presence of appropriate transcription factors, as demonstrated by the expression of the active alleles. This phenomenon resembles the situation found in genomic imprinting, in which methylation plays a key role (Jaenisch 1997; Li et al. 1993). In each case, the mere presence of transcription factors is insufficient to induce transcription. The *RT6* promoter is also active in *RT6*-nonexpressing rat1 fibroblasts, C58NT, and Cos7 cells (Haag et al. 1996; results not shown). The correct expression of tissue-specific genes requires the coordinated interplay between the transcription machinery and factors determining the chromatin structure (Bonifer 2000). The finding that methyl-DNA-binding proteins direct histone deacetylases to methylated DNA and thereby repress opening of chromatin (Jones et al. 1998; Nan et al. 1998) provides an explanation as to how DNA methylation contributes to *RT6* gene regulation.

Monoallelic expression, presumably due to distinct mechanisms, is observed in a growing group of genes. In some of these, selection of the expressed allele occurs in a stochastic fashion. Examples include genes for olfactory receptors (Chess et al. 1994), immunoglobulin, T-cell receptor (Mostoslavsky and Bergman 1997), Ly-49 natural killer cell receptors (Held and Kunz 1998), interleukin-2 (Hollander et al. 1998), interleukin-4 (Bix and Locksley 1998), and Pax5 (Nutt et al. 1999), as well as in X chromosome inactivation (Mostoslavsky and Bergman 1997). In these instances, sequence differences cannot be held accountable for differential expression of the alleles. In genomic imprinting, the parental origin determines which allele is transcribed (Jaenisch 1997). In monoallelic expression of imprinted genes (Li et al. 1993) and in allelic exclusion of immunoglobulin genes (Mostoslavsky et al. 1998), a role for DNA methylation has been established. In contrast to the phenomena described above, monoallelic expression of *RT6* is determined by allele-inherent *cis*-regulatory elements. A similar situation is also observed for mouse *Rt6* (Art2), where two closely related genes exist. Strain-dependent differences in *Rt6* expression are observed, and are regulated by *cis*-acting mechanisms (Koch-Nolte et al. 1999; Sardinha and Rajan 1999). Other genes that show allele-determined expression differences include those for CD44 (Lynch and Ceredig 1989), the activation antigen GL7 (Hathcock et al. 1995), Thy-1 (Spangrude and Brooks 1992) as well as the genes of the Ly-6 complex Ly-6A/E (Spangrude and Brooks 1993), Ly-6C (Schlueter et al. 1997) and ThB (Gumley et al. 1994). It is particularly interesting that the genes of the Ly-6 complex are tightly linked within a region comprising 650 kb (Gumley et al. 1995). Mice with the Ly-6.2 haplotype generally express the alloantigens in a higher proportion of cells than do mice with the Ly-6.1 haplotype. We speculate that the expression differences observed in the above cases are due to allele-specific differences in DNA methylation status and chromatin structure, rendering the genes inaccessible to transactivating factors.

The results obtained from the promoter-specific in vitro methylation indicate that the sequence context

of the methylated sites plays an important role in mediating the repressive effect of methylation on *RT6* gene activity. While *RT6* promoter activity was inhibited by methylation, no effect was observed on SV40 promoter activity. This is remarkable, given the higher density of CpG dinucleotides in the SV40 promoter (5 sites/100 bp) than in the *RT6* promoter (1.4 sites/100 bp). The observed insensitivity of the SV40 promoter to methylation is consistent with the finding that methylation of SV40 does not affect its transformation efficiency, viral replication or expression of T and V antigens (Graessmann et al. 1983). Two of the four CpG dinucleotides in the *RT6* promoter lie in predicted transcription factor-binding sites (Haag et al. 1996). These comprise two predicted c-Myb-binding sites at positions –1 to +8 and –44 to –36 relative to the transcription start site, the latter binding site overlaping with a site for c-Myc. Intriguingly, binding of both c-Myb (Klempnauer 1993) and c-Myc (Prendergast et al. 1991) to their target sequences is sensitive to DNA methylation. Therefore, methylation of these sites could explain the observed inhibitory effects of methylation on *RT6* promoter activity. In vivo, however, larger regions of the *RT6* gene are methylated. Inhibition of transcription factor binding probably contributes synergistically with other methylation-dependent factors such as MeCP2, in concert with chromatin remodeling, to the silencing of the *RT6* gene by methylation.

Monoallelic *RT6* expression has been observed in T-cell subpopulations of all *RT6a*×*RT6b* crossbreeds investigated and is independent of age and sex (Crisa et al. 1990; Thiele et al. 1993). Since monoallelic *RT6* expression is also observed in crosses between LEW (*RT6a*) and congenic LEW.6B (*RT6b*) rats that are genetically identical except for their *RT6* locus (Wonigeit 1979), one or several *cis*-regulating elements in the *RT6* gene or close to it must be crucial for the demethylation process. Our results indicate that this asymmetric expression is due to a failure to demethylate the *RT6a* locus in the RT6.2 single-positive T-cell population of F1 rats. Allelic expression differences are not restricted to heterozygous rats but are also observed if *RT6* homozygous rats are compared. *RT6b* rats consistently possess a higher percentage of *RT6*-expressing T cells than *RT6a* rats (Thiele et al. 1993). The two known *RT6* alleles are highly divergent, their gene products differing in 10 of 226 amino acids. Indeed, there are more nonsynonymous than synonymous mutations (Haag et al. 1990b), suggesting that the polymorphism at the *RT6* locus may be maintained by selection (Howard 1997). This suggestion is strengthened by the observation that the two alloantigens show different enzyme activities (Haag et al. 1995; Hara et al. 1996). Differential regulation of *RT6* alleles itself has phenotypic consequences and may also result from selection. Since the precise function of the RT6 ADP-ribosyltransferases within the immune system of the rat is not yet known, the biological significance of the polymorphism at this locus remains unclear.

Our data suggest that restriction of *RT6* expression to a late stage in T-cell development is regulated by DNA methylation. The finding that this process is regulated in an allele-specific fashion in *RT6* congenic rat strains shows that *cis*-regulatory elements are involved, and make the *RT6* gene an attractive system to study the mechanisms controlling methylation status. The *RT6* transcription/demethylation control region may prove useful for promoting specific expression in mature T cells in transgenic animals and conditional knockout mice. This system could overcome the shortcoming of the T cell-specific promoters currently available, which are already active in the thymus and are therefore only partially suitable for the study of late T-cell development. Availability of a promoter specific for mature T cells would provide a valuable tool for studying the immune system. Finally, our data draw attention to the possibility that allelic differences in elements that control methylation and demethylation processes, in concert with allelic variations in the coding and regulatory regions of genes, may contribute to genetic variation and susceptibility to diseases such as cancer and autoimmune disorders.

Acknowledgements We thank Katrin Bartels for excellent technical assistance and Ky Lowenhaupt, Thomas Schwartz, and Detlev Biniszkiewicz for critical reading of this manuscript and helpful discussions. Essential parts of this work are part of the doctoral thesis of S.R., who is a fellow of the Werner-Otto-Stiftung. This work was supported by grant DFG Ha2369/1 to F.H. and H.-G.T. The experiments performed comply with the laws of the Federal Republic of Germany.

References

Antequera F, Bird A (1993) CpG islands. In: Jost JP, Saluz HP (eds) DNA methylation: biochemistry and biological significance. Birkhäuser, Basel, pp 169–185

Bird AP (1995) Gene number, noise reduction and biological complexity. Trends Genet 11:94–100

Bix M, Locksley RM (1998) Independent and epigenetic regulation of the interleukin-4 alleles in CD4+ T cells. Science 281:1352–1354

Bonifer C (2000) Developmental regulation of eukaryotic gene loci: which cis-regulatory information is required? Trends Genet 16:310–315

Bourbon H-M, Prudhomme M, Amalric F (1988) Sequence and structure of the nucleolin promoter in rodents: characterization of a strikingly conserved CpG island. Gene 68:73–84

Chess A, Simon I, Cedar H, Axel R (1994) Allelic inactivation regulates olfactory receptor gene expression. Cell 78:823–834

Clevers HC, Grosschedl R (1996) Transcriptional control of lymphoid development: lessons from gene targeting. Immunol Today 17:336–343

Crisa L, Greiner DL, Mordes JP, MacDonald RG, Handler ES, Czech MP, Rossini AA (1990) Biochemical studies of RT6 alloantigens in BB/Wor and normal rats: evidence for intact unexpressed RT6a structural gene in diabetes-prone BB rats. Diabetes 39:1279–1288

Doerfler W (1991) Patterns of DNA methylation – evolutionary vestiges of foreign DNA inactivation as a host defense mechanism. A proposal. Biol Chem Hoppe Seyler 372:557–564

Ehrlich M, Ehrlich KC (1993) Effect of DNA methylation on the binding of vertebrate and plant proteins to DNA. In: Jost JP, Saluz HP (eds) DNA methylation: biochemistry and biological significance. Birkhäuser, Basel, pp 145–168

Fangmann J, Schwinzer R, Hedrich HJ, Kloting I, Wonigeit K (1991) Diabetes-prone BB rats express the RT6 alloantigen on intestinal intraepithelial lymphocytes. Eur J Immunol 21:2011–2015

Graessmann M, Graessmann A (1993) DNA methylation, chromatin structure and the regulation of gene expression. In: Jost JP, Saluz HP (eds) DNA methylation: biochemistry and biological significance. Birkhäuser, Basel, pp 404–424

Graessmann M, Graessmann A, Wagner H, Werner E, Simon D (1983) Complete DNA methylation does not prevent polyoma and simian virus 40 virus early gene expression. Proc Natl Acad Sci USA 80:6470–6474

Greiner DL, Mordes JP, Handler ES, Angelillo M, Nakamura N, Rossini AA (1987) Depletion of RT6.1+ T lymphocytes induces diabetes in resistant biobreeding/Worcester (BB/W) rats. J Exp Med 166:461–475

Gumley TP, McKenzie IF, Sandrin MS (1994) Polymorphism at the mouse Thb locus. Immunogenetics 39:390–394

Gumley TP, McKenzie IF, Sandrin MS (1995) Tissue expression, structure and function of the murine Ly-6 family of molecules. Immunol Cell Biol 73:277–296

Haag F, Koch-Nolte F (1998) Endogenous relatives of ADP-ribosylating bacterial toxins in mice and men: potential regulators of immune cell function. J Biol Regul Homeost Agents 12:53–62

Haag F, Koch F, Thiele HG (1990a) Nucleotide and deduced amino acid sequence of the rat T-cell alloantigen RT6.1. Nucleic Acids Res 18:1047

Haag F, Koch F, Thiele HG (1990b) Polymorphism between rat T-cell alloantigens RT6.1 and RT6.2 is based on multiple amino acid substitutions. Transplant Proc 22:2541–2542

Haag F, Andresen V, Karsten S, Koch-Nolte F, Thiele HG (1995) Both allelic forms of the rat T cell differentiation marker RT6 display nicotinamide adenine dinucleotide (NAD)-glycohydrolase activity, yet only RT6.2 is capable of automodification upon incubation with NAD. Eur J Immunol. 25:2355–2361

Haag F, Kuhlenbäumer G, Koch-Nolte F, Wingender E, Thiele HG (1996) Structure of the gene encoding the rat T cell ecto-ADP-ribosyltransferase RT6. J Immunol 157:2022–2030

Haag F, Rothenburg S, Koch-Nolte F, Thiele HG (1997) Polymorphic microsatellite within the main promoter of the rat T cell differentiation antigen RT6 on chromosome 1. Transplant Proc 29:1703–1704

Hara N, Tsuchiya M, Shimoyama M (1996) Glutamic acid 207 in rodent T-cell RT6 antigens is essential for arginine-specific ADP-ribosylation. J Biol Chem 271:29552–29555

Hathcock KS, Pucillo CE, Laszlo G, Lai L, Hodes RJ (1995) Analysis of thymic subpopulations expressing the activation antigen GL7: expression, genetics, and function. J Immunol 155:4575–4581

Held W, Kunz B (1998) An allele-specific, stochastic gene expression process controls the expression of multiple Ly49 family genes and generates a diverse MHC-specific NK cell receptor repertoire. Eur J Immunol 28:2407–2416

Hohn T, Corsten S, Rieke S, Muller M, Rothnie H (1996) Methylation of coding region alone inhibits gene expression in plant protoplasts. Proc Natl Acad Sci USA 93:8334–8339

Hollander GA, Zuklys S, Morel C, Mizoguchi E, Mobisson K, Simpson S, Terhorst C, Wishart W, Golan DE, Bhan AK, Burakoff SJ (1998) Monoallelic expression of the interleukin-2 locus. Science 279:2118–2121

Howard JC (1997) Contemplations on the evolution of pro- and eukaryotic mono(ADP-ribosyl) transferases in the context of the immune system. In: Haag F, Koch-Nolte F (eds) ADP-ribosylation in animal tissues: structure, function, and biology of mono(ADP-ribosyl)transferases and related enzymes. Plenum, New York, pp 453–458

Jaenisch R (1997) DNA methylation and imprinting: why bother? Trends Genet 13:323–329

Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, Wolffe AP (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 19:187–191

Klempnauer KH (1993) Methylation-sensitive DNA binding by v-myb and c-myb proteins. Oncogene 8:111–115

Koch F, Kashan A, Thiele HG (1988) Production of a rat T cell hybridoma that stably expresses the T cell differentiation marker RT6.2. Hybridoma 7:341–353

Koch F, Haag F, Kashan A, Thiele HG (1990) Primary structure of rat RT6.2, a nonglycosylated phosphatidylinositol-linked surface marker of postthymic T cells. Proc Natl Acad Sci USA 87:964–967

Koch-Nolte F, Klein J, Hollmann C, Kühl M, Haag F, Gaskins R, Leiter E, Thiele HG (1995) Defects in the structure and expression of the genes for the T cell marker *Rt6* in NZW and (NZB×NZW) F_1 mice. Int Immunol 7:883–890

Koch-Nolte F, Duffy T, Nissen M, Kahl S, Killeen N, Ablamunits V, Haag F, Leiter EH (1999) A new monoclonal antibody detects a developmentally regulated mouse ecto-ADP-ribosyltransferase on T cells: subset distribution, inbred strain variation, and modulation upon T cell activation. J Immunol 163:6014–6022

Li E, Beard C, Jaenisch R (1993) Role for DNA methylation in genomic imprinting. Nature 366:362–365

Lynch F, Ceredig R (1989) Mouse strain variation in Ly-24 (Pgp-1) expression by peripheral T cells and thymocytes: implications for T cell differentiation. Eur J Immunol 19:223–229

Matthes M, Hollmann C, Bertuleit H, Kühl M, Thiele HG, Haag F, Koch-Nolte F (1997) 'Natural' Rt6-1 and Rt6-2 'knock-out' mice. In: Haag F, Koch-Nolte F (eds) ADP-ribosylation in animal tissues: structure, function, and biology of mono(ADP-ribosyl)transferases and related enzymes. Plenum, New York, pp 271–274

Mostoslavsky R, Bergman Y (1997) DNA methylation: regulation of gene expression and role in the immune system. Biochim Biophys Acta 1333:F29–F50

Mostoslavsky R, Singh N, Kirillov A, Pelanda R, Cedar H, Chess A, Bergman Y (1998) Kappa chain monoallelic demethylation and the establishment of allelic exclusion. Genes Dev 12:1801–1811

Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393:386–389

Nemoto E, Yu Y, Dennert G (1996) Cell surface ADP-ribosyltransferase regulates lymphocyte function- associated molecule-1 (LFA-1) function in T cells. J Immunol 157:3341–3349

Newell-Price J, Clark AJ, King P (2000) DNA methylation and silencing of gene expression. Trends Endocrinol Metab 11:142–148

Nutt SL, Vambrie S, Steinlein P, Kozmik Z, Rolink A, Weith A, Busslinger M (1999) Independent regulation of the two Pax5 alleles during B-cell. Nat Genet 21:390–395

Okamoto S, Azhipa O, Yu Y, Russo E, Dennert G (1998) Expression of ADP-ribosyltransferase on normal T lymphocytes and effects of nicotinamide adenine dinucleotide on their function. J Immunol 160:4190–4198

Okazaki IJ, Moss J (1998) Glycosylphosphatidylinositol-anchored and secretory isoforms of mono-ADP-ribosyltransferases. J Biol Chem 273:23617–23620

Prendergast GC, Lawe D, Ziff EB (1991) Association of Myn, the murine homolog of max, with c-Myc stimulates methylation-sensitive DNA binding and ras cotransformation. Cell 65:395–407

Rothenburg S, Koch-Nolte F, Thiele HG, Haag F (1999a) Evidence that allelic variations in the main RT6 promoter modulate transcriptional activity. Transplant Proc 31:1626–1628

Rothenburg S, Koch-Nolte F, Thiele HG, Haag F (1999b) Expression of the T-cell differentiation antigen RT6.2 in T-cell lines is correlated with hypomethylation of the RT6 promoter. Transplant Proc 31:1624–1625

Sardinha DF, Rajan TV (1999) Cis-acting regulation of splenic Art2 gene expression in inbred mouse strains. Immunogenetics 49:700–703

Schlueter AJ, Malek TR, Hostetler, CN, Smith PA, deVries P, Waldschmidt TJ (1997) Distribution of Ly-6C on lymphocyte subsets. I. Influence of allotype on T lymphocyte expression. J Immunol 158:4211–4222

Singal R, Ferris R, Little JA, Wang SZ, Ginder GD (1997) Methylation of the minimal promoter of an embryonic globin gene silences transcription in primary erythroid cells. Proc Natl Acad Sci USA 94:13724–13729

Singer-Sam J, Grant M, LeBon JM, Okuyama K, Chapman V, Monk M, Riggs AD (1990) Use of a *Hpa*II-polymerase chain reaction assay to study DNA methylation in the Pgk-1 CpG island of mouse embryos at the time of X-chromosome inactivation. Mol Cell Biol 10:4987–4989

Spangrude GJ, Brooks DM (1992) Phenotypic analysis of mouse hematopoietic stem cells shows a Thy-1-negative subset. Blood 80:1957–1964

Spangrude GJ, Brooks DM (1993) Mouse strain variability in the expression of the hematopoietic stem cell antigen Ly-6A/E by bone marrow cells. Blood 82:3327–3332

Tajima S, Suetake I (1998) Regulation and function of DNA methylation in vertebrates. J Biochem (Tokyo) 123:993–999

Thiele HG (1988) Rat Thy-1 antigen and its relation to RT6. In: Reif AE, Schlesinger M (eds) Cell surface antigen Thy-1: immunology, neurology, and therapeutic applications. Dekker, New York, pp 149–170

Thiele HG, Haag F, Nolte F (1993) Asymmetric expression of RT6.1 and RT6.2 alloantigens in (RT6a×RT6b)F1 rats is due to a pretranslational mechanism. Transplant Proc 25:2786–2788

Walsh CP, Bestor TH (1999) Cytosine methylation and mammalian development. Genes Dev 13:26–34

Wonigeit K (1979) Characterization of the RT-Ly-1 and RT-Ly-2 alloantigenic systems by congenic rat strains. Transplant Proc 11:1631–1635

Wonigeit K, Dinkel A, Fangmann J, Thude H (1997) Expression of the ectoenzyme RT6 is not restricted to resting peripheral T cells and is differently regulated in normal peripheral T cells, intestinal IEL, and NK cells. In: Haag F, Koch-Nolte F (eds) ADP-ribosylation in animal tissues: structure, function, and biology of mono(ADP-ribosyl)transferases and related enzymes. Plenum, New York, pp 229–240

Yeivin A, Razin A (1993) Gene methylation patterns and expression. In: Jost JP, Saluz HP (eds) DNA methylation: biochemistry and biological significance. Birkhäuser, Basel, pp 523–568

Yoder JA, Walsh CP, Bestor TH (1997) Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 13:335–340